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PanG, a New Ketopantoate Reductase Involved in Pantothenate Synthesis

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Pantothenate, commonly referred to as vitamin B_5, is an essential molecule in the metabolism of living organisms and forms the core of coenzyme A. Unlike humans, some bacteria and plants are capable of *de novo* biosynthesis of pantothenate, making this pathway a potential target for drug development. *Francisella tularensis* subsp. *tularensis* Schu S4 is a zoonotic bacterial pathogen that is able to synthesize pantothenate but is lacking the known ketopantoate reductase (KPR) genes, *panE* and *ilvC*, found in the canonical *Escherichia coli* pathway. Described herein is a gene encoding a novel KPR, for which we propose the name *panG* (FTT1388), which is conserved in all sequenced *Francisella* species and is the sole KPR in Schu S4. Homologs of this KPR are present in other pathogenic bacteria such as *Enterococcus faecalis*, *Coxiella burnetii*, and *Clostridium difficile*. Both the homologous gene from *E. faecalis* V583 (EF1861) and *E. coli panE* functionally complemented *Francisella novicida* lacking any KPR. Furthermore, *panG* from *F. novicida* can complement an *E. coli* KPR double mutant. A Schu S4 Δ*panG* strain is a pantothenate auxotroph and was genetically and chemically complemented with *panG* in *trans* or with the addition of pantolactone. There was no virulence defect in the Schu S4 Δ*panG* strain compared to the wild type in a mouse model of pneumonic tularemia. In summary, we characterized the pantothenate pathway in *Francisella novicida* and *F. tularensis* and identified an unknown and previously uncharacterized KPR that can convert 2-dehydropantoate to pantoate, PanG.

Coenzyme A (CoA) is a ubiquitous cofactor found in all domains of life and plays a central role in carbon and energy metabolism. Pantothenate forms the core of CoA; however, animals and some microorganisms lack the ability to synthesize pantothenate. The pantothenate biosynthetic pathway is present in some plants, fungi, bacteria, and archaea, making the pathway a strong candidate for the discovery of novel antibiotics (1). The pantothenate biosynthesis pathway was first genetically and chemically defined in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium and is composed of four enzymes that use the precursors 2-oxoisovalerate and L-aspartic acid to make pantothenate (2, 3) (Fig. 1A). In one branch of the pathway, aspartate-1-decarboxylase, PanD, converts L-aspartate to β-alanine, and in a separate branch two enzymes, PanB, a ketopantoate hydroxymethyltransferase, and PanE, a ketopantoate reductase (KPR), convert 2-oxoisovalerate to pantoate (4). The acetohydroxy acid isomerase, IlvC, has also been shown to function as a KPR in *E. coli* and *S.* Typhimurium, converting 2-dehydropantoate to pantoate, similar to PanE (5, 6). In some species, e.g., *Corynebacterium glutamicum*, IlvC is the sole KPR (7). In the final step of the pantothenate synthesis pathway, pantoate and β-alanine are ligated by the pantothenate synthase, PanC, to form pantothenate (8). The published genomes of *Francisella* species contain *panB*, *panC*, and *panD*. No homolog of *E. coli* PanE exists in the sequenced *Francisella* genomes, and the most virulent strain of *Francisella tularensis*, strain Schu S4, contains a frameshift mutation in *ilvC*, suggesting a gap in the pantothenate biosynthetic pathway. Combining the known nutritional requirements of *Francisella* species with the sequenced genomes revealed that several strains require exogenous pantothenate for growth (9). Interestingly, the most virulent strain Schu S4 lacks a recognizable KPR and is a pantothenate prototroph, while the live vaccine strain (LVS) has a KPR but is clearly auxotrophic (10, 11). The genetic basis for differences in the requirement for pantothenate among *Francisella* strains is not known.

Francisella belongs to the *Gammaproteobacteria* and includes a number of species that share greater than 95% genomic similarity but differ in their ability to cause the disease tularemia in humans (12, 13). Of all the species, *Francisella tularensis* subsp. *tularensis* is the most virulent, exhibiting the highest morbidity and mortality while also having a very low infectious dose, less than 10 bacteria (11, 14). *F. tularensis* subsp. *holarctica* is also highly infectious for humans, but clinical disease associated with this bacterium is typically mild (15). The LVS was derived from an *F. tularensis* subsp. *holarctica* strain and is attenuated in humans. *Francisella novicida* is rarely associated with human disease. As a facultative intracellular pathogen, *Francisella* must acquire the nutrients needed to support growth while within host cells. Several genetic screens for replication-defective mutants have implicated biosynthetic pathways as contributing factors to the pathogenesis of *Francisella* species (16). Interruption in genes involved in purine, aromatic amino acid, and biotin biosynthesis results in attenuation of *F. tularensis* within a mouse model of tularemia (17–20). Collectively, these studies highlight the contribution of *de novo* biosynthetic pathways to the ability of *F. tularensis* to survive within the host environment. Using *F. novicida*, a genome-wide mutant screen identified two genes contained in the putative pantothenate operon that are required for pulmonary and systemic infections in mice. Inactivation of FTN_1351 (*panG*) or FTN_1355

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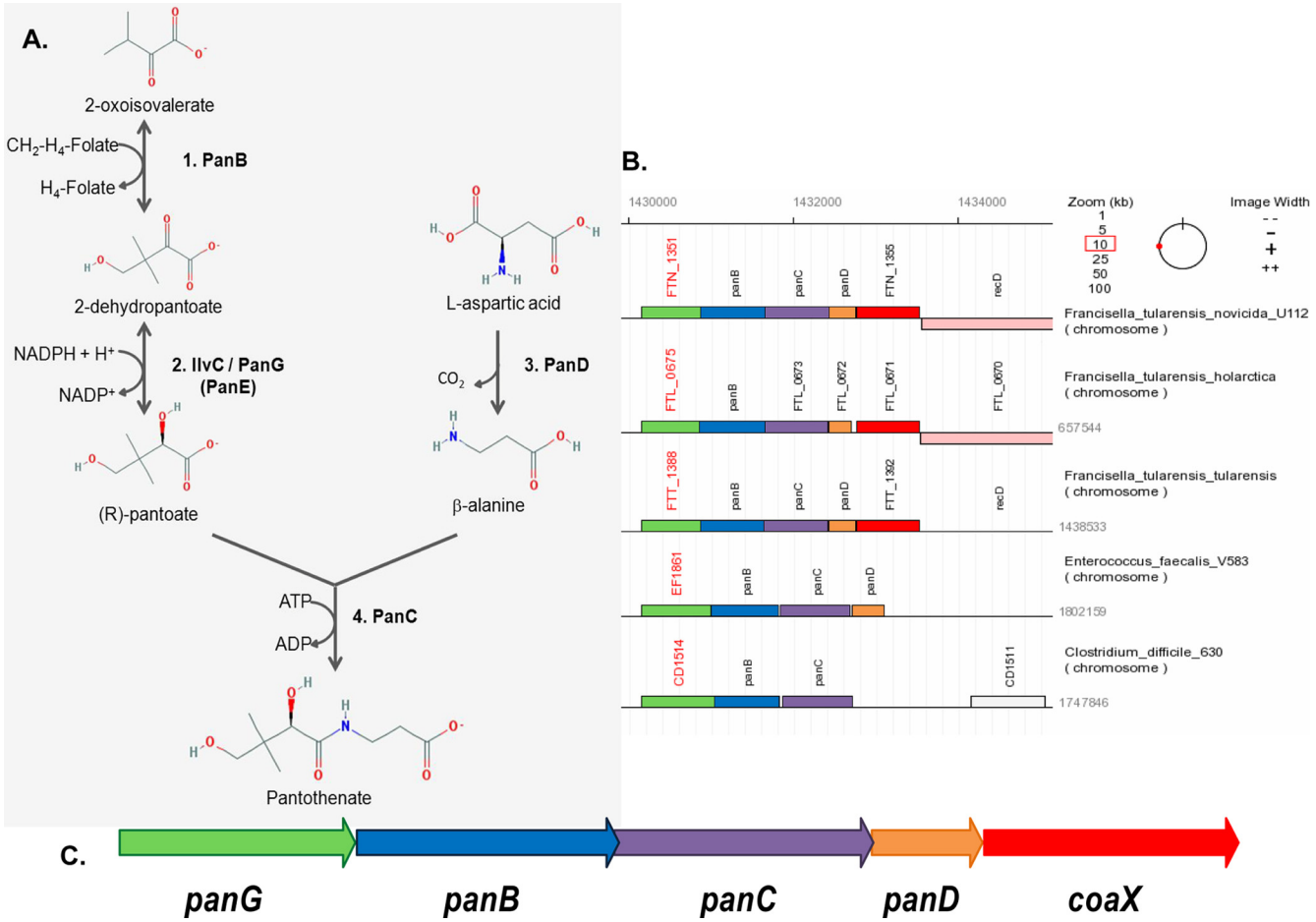

FIG 1 The biosynthetic pathway in *Francisella* species and the putative pantothenate operon. (A) The pantothenate biosynthetic pathway consists of two converging arms. PanB, the ketopantoate hydroxymethyltransferase, converts 2-oxoisovalerate with tetrahydrofolate to form 2-dehydropantoate. The substrate 2-dehydropantoate is then converted to (R)-pantoate by a number of enzymes, including IlvC and PanG, which are both capable of ketopantoate reductase (KPR) activity. On the other branch of the pathway, PanD, an aspartate-1-decarboxylase, converts L-aspartic acid to β-alanine. The pathway converges with PanC, the pantothenate synthase that ligates (R)-pantoate with β-alanine to form pantothenate. Molecules were made in PubChem Compound on NCBI, and the pathway was constructed using KEGG metabolic pathway 00770 as a reference (http://www.genome.jp/kegg-bin/show_pathway?FTN_00770). (B) The genomic organization of the putative pantothenate operon is conserved among sequenced *Francisella* strains containing the *panGBCD* genes and the pantothenate kinase gene *coaX*. (C) Synteny diagram of the putative operon in *F. novicida*, *F. tularensis* LVS, *F. tularensis* Schu S4, *E. faecalis* V583, and *C. difficile* 630 (35).

(*coaX*) resulted in mutant strains impaired in their ability to proliferate in the organs of infected mice (21). Furthermore, transcriptional profiling of *F. tularensis* revealed a dramatic increase in expression of the *F. tularensis* homologs of *panB*, *panC*, and *panD*, as well as the homologs of FTN_1351 (*panG*) and FTN_1355 (*coaX*) in strains grown within bone marrow-derived macrophages. This suggests that pantothenate biosynthesis may play a role in the adaptation of *Francisella* to its intracellular niche (22). The requirement for *de novo* synthesis of pantothenate in virulence was demonstrated in a different intracellular pathogen, *Mycobacterium tuberculosis*, for which a double mutant in *panC* and *panD* was attenuated in both BALB/c and SCID mouse models of infection (23). Collectively the published data suggest that pantothenate biosynthesis may contribute to the virulence of *Francisella*, and the disparity in the requirement for pantothenate among *Francisella* strains led us to investigate the genetic basis for pantothenate biosynthesis in several species of *Francisella*.

MATERIALS AND METHODS

Bacterial strains and cell culture. *Escherichia coli* strains (Table 1) were grown at 37°C in Luria-Bertani (LB) broth, on LB agar, or in M9 minimal medium supplemented with 1 μg/ml thiamine, 3.4 mM valine, 3 mM isoleucine, and 3 mM leucine. Unless otherwise noted, all *F. tularensis* strains (Table 1) were grown at 37°C on chocolate agar (25 g brain heart infusion [BHI] liter^{-1}, 10 μg hemoglobin liter^{-1}, 15 g agar liter^{-1}) supplemented with 1% IsoVitalex (Becton-Dickson) or in Chamberlain's Defined Medium (CDM) (10). When necessary, hygromycin B (Hyg; Roche Applied Science) was used at 200 μg ml^{-1}, sucrose was used at 5% for *E. coli* or 10% for *F. tularensis* (wt/vol), ampicillin (Amp; Roche Applied Science) was used at 100 μg ml^{-1}, L-arabinose (Sigma-Aldrich) was used at 10 mM, and kanamycin (Km; Sigma-Aldrich) was used at 50 μg ml^{-1} for *E. coli* and 10 μg ml^{-1} for *F. tularensis*.

Growth assays. Growth assays for *F. tularensis* and *F. novicida* were performed at 37°C while shaking in an Infinite M200 or M200 pro (Tecan) apparatus in 96-well microtiter plates with absorbance (optical density at 600 nm [OD_{600}]) monitored every 15 min. Bacterial strains grown over-

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source
Bacterial strains		
E. coli DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara-leu*) *7697 galU galK rpsL nupG* λ$^-$	Invitrogen
E. coli Top10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*) *7697 galU galK rpsL* (Str^r) *endA1 nupG* λ$^-$	Invitrogen
E. coli panE::Kan JW0415-1	Δ*(araD-araB)567* Δ*lacZ4787(::rrnB-3)* Δ*panE782*::Kan λ$^-$ *rph*-Δ*(rhaD-rhaB)568 hsdR514*	Keio Collection
E. coli ilvC::Kan JW3747-2	Δ*(araD-araB)567*, Δ*lacZ4787(::rrnB-3)* λ$^-$, *rph-1*, Δ*ilvC725*::Kan Δ*(rhaD-rhaB)568 hsdR514*	Keio Collection
F. novicida U112	*F. tularensis* subsp. *novicida* U112 (taxid, 401614)	ATCC
F. holarctica LVS	*F. tularensis* subsp. *holarctica* LVS (taxid, 376619)	CDC
F. tularensis Schu S4	*F. tularensis* subsp. *tularensis* SCHU S4 (taxid, 177416)	BEI Resources
E. faecalis	Strain V583	Lance Thurlow
F. tularensis		
panB::Tn strain	tnfn1_pw060328p05q156 T20 (IS*Fn2*/FRT) (FTN_1352)	24
panC::Tn strain	tnfn1_pw060328p03q178 T20 (IS*Fn2*/FRT) (FTN_1353)	24
panD::Tn strain	tnfn1_pw060323p04q175 <Kan-2> (FTN_1354)	24
panG::Tn strain	tnfn1_pw060420p03q142 T20 (IS*Fn2*/FRT) (FTN_1351)	24
ilvC::Tn strain	tnfn1_pw060420p04q122 T20 (IS*Fn2*/FRT) (FTN_1040)	24
FTN_1698::Tnflp[a] strain	tnfn1_pw06032p08q164	This work
ilvC::Tnflp[a] *panG*::Tn strain	tnfn1_pw060420p04q122flp/tnfn1_pw060420p03q142	This work
SKI01	*F. tularensis* Schu S4 Δ*panG*	This work
SKI02	*F. novicida* U112 *ilvC*::Tnflp *panG*::Tn with pEDL70	This work
SKI03	*F. novicida* U112 *ilvC*::Tnflp *panG*::Tn with pEDL71	This work
SKI04	*F. novicida* U112 *ilvC*::Tnflp *panG*::Tn with pSKI01	This work
SKI05	*F. tularensis* Schu S4 Δ*panG* with pSKI01	This work
SKI06	*F. tularensis* Schu S4 Δ*panG* with pMP831 empty vector control	This work
SKI07	*F. novicida* U112 *ilvC*::Tnflp *panG*::Tn with pMP831 empty vector control	This work
SKI08	*F. holarctica* LVS with *panD* from *F. novicida* U112	This work
SKI09	*E. coli ilvC*::Flp *panE*::Kan (JW0415-1 and JW3747-2)	This work
SKI10	*E. coli ilvC*::Flp *panE*::Kan (JW0415-1 and JW3747-2) with pSKI01	This work
SKI11	*E. coli ilvC*::Flp *panE*::Kan (JW0415-1 and JW3747-2) with pSKI04	This work
Plasmids		
pFFLP-hyg	Carries *flp* under *Francisella groEL* promoter for recombination of FRT sites, TempSens, Hyg^r	25
pKD46	λ-Red recombineering plasmid, GenBank accession no. J02459.1, TempSens, Amp^r	26
pFlp2	Carries *flp* recombinase for recombination of FRT sites, Amp^r, Suc^s	27
pEDL70	pMP822.*panE*, Hyg^r	This work
pEDL71	pMP822.EF1861, Hyg^r	This work
pSKI01	pMP831.*panG* FTN_1351, Hyg^r	This work
pSKI02	pEDL50 with Δ*panG* Schu S4 construct, Km^r, Suc^s	This work
pSKI03	pMP590 with $panD_{U112}$ (FTN_1354), Km^r, Suc^s	This work
pSKI04	pMP822.*ilvC*, Hyg^r	This work
pMP590	*sacB* suicide vector, Km^r, Suc^s	28
pMP822	*E. coli-F. tularensis* shuttle vector, Hyg^r, *blaB* promoter	29
pMP831	*E. coli-F. tularensis* shuttle vector, Hyg^r	29
pEDL50	Conjugative *sacB* suicide vector, Hyg^r, Suc^s	30

[a] "flp" signifies a cured kanamycin cassette.

night on chocolate agar were resuspended in phosphate-buffered saline (PBS) to a Klett reading of 100 (approximately 1×10^9 CFU/ml). Resuspended cultures were diluted 1:20 into test media. The test medium consisted of CDM or of CDM without calcium pantothenate (Sigma-Aldrich). Genetic complementation in *trans* was evaluated in CDM lacking pantothenate. Chemical complementation was evaluated in CDM lacking pantothenate and supplemented with 100 μM pantolactone (Sigma-Aldrich), which can act in place of pantoate (31), or 100 μM β-alanine (Sigma-Aldrich).

Construction of a double mutant using an Flp/Frt recombination in *F. novicida*. To create the *ilvC*::Tnflp *panG*::Tn mutant ("flp" signifies a cured Km cassette) we performed Flp-mediated recombination of *ilvC*::Tn bearing the T20 insertion by introducing pFFlp-hyg, a temperature-sensitive plasmid, by electroporation and isolated Km-sensitive colonies grown at 30°C (25). Removal of the Km cassette left an 80-bp insertion with the expected single FLP recombination target (FRT) site remaining. Once the Km resistance marker was excised, pFFlp-hyg was cured by growing the culture overnight at 37°C. The *panG* mutation was introduced by transformation of genomic DNA from the *panG*::Tn strain into the *ilvC*::Tnflp mutant and selecting for Km-resistant colonies, creating the double mutant (25). For genetic complementation, mutant strains were transformed with their respective plasmids (29). Plasmids were first transformed with the addition of 1 μl of TypeOne Restriction Inhibitor (Epicentre) into an FTN_1698::Tnflp restriction mutant strain (25).

Construction of the *E. coli ilvC*::Flp *panE*::Kan double mutant using FLP/FRT recombination and λ-Red recombineering. Using λ-Red recombineering, the *ilvC*::flp *panE*::Kan double mutant ("flp" signifies a cured Km cassette) was created in *E. coli* by first introducing pFlp2 into *ilvC*::Kan (JW3747-2) by electroporation and isolating Km-sensitive col-

onies (26, 32). Removal of the kanamycin cassette left the signature single FRT site of FLP/FRT recombination, creating *ilvC*::Flp. Once the Km resistance marker was excised, pFlp2 was cured by growing the culture overnight and plating on LB with 5% sucrose and without NaCl, and the resulting colonies were screened for Amp sensitivity. Next, the λ-Red recombineering plasmid pKD46 was introduced into the *ilvC*::Flp strain by electroporation (27). When the absorbance of *ilvC*::Flp with pKD46 reached an OD_{600} of 0.1, 10 mM L-arabinose was added to induce expression of the λ-Red recombineering proteins: Gam, Bet, and Exo. The *panE*::Kan mutation was introduced by transformation into the *ilvC*::Flp mutant using PCR fragments generated from genomic DNA of *panE*::Kan (JV0415-1), and colonies were selected for Km resistance.

***F. novicida* CoA levels.** *F. novicida* was grown overnight on chocolate agar plates at 37°C and then resuspended to an OD_{600} of 0.2 in 50 ml of CDM lacking pantothenate and incubated for 5 h. The cells were pelleted at 10,000 × *g* for 5 min at 4°C and then resuspended in 0.5 ml of cold PBS and snap-frozen in liquid nitrogen. The cells were thawed and bead beaten 2 times for 30 s at 4°C using lysing matrix B tubes (MP Biomedicals). Cell debris was removed by centrifuging at 14,000 × *g* for 2 min. Protein concentration was determined by bicinchoninic acid (BCA) assay for each sample. To deproteinize the lysates, 100 μl of cold 1 N perchloric acid was added to each sample and centrifuged at 14,000 × *g* at 4°C for 2 min to pellet precipitated protein. To neutralize the supernatant, 30 μl of cold 3 M potassium bicarbonate was added to the samples. The concentrations of CoA were determined for each sample using the PicoProbe Acetyl-CoA assay kit (BioVision). The manufacturer's protocol was followed, except that the CoA quenching solution was not used, thereby allowing us to determine the total concentration of CoA in each sample.

Replacing LVS *panD* ($panD_{LVS}$) with *F. novicida panD* ($panD_{U112}$). The putative *panD* (FTN_1354) allele was PCR amplified from *F. novicida* genomic DNA. The amplified DNA was subcloned into the TOPO pCRII vector (Invitrogen) for propagation and maintenance. The BamHI-NotI fragment containing the *F. novicida panD* allele was ligated into the *sacB* suicide vector pMP590 and electroporated into LVS. Allelic exchange was performed as previously described (28).

Deletion of FTT1388 (*panG*) in Schu S4. The putative *panG* (FTT1388) allele plus 300 bp of DNA flanking each end was PCR amplified from *F. tularensis* Schu S4 genomic DNA. The amplified fragment was cut with BamHI and NotI and then ligated into the *sacB* suicide vector, pEDL50 (30). Using splice junction PCR, FTT1388 (*panG*) was eliminated from the plasmid, creating a product with AatII restriction sites on either end. The PCR product was digested with AatII and ligated, forming pSKI01. Conjugation and allelic exchange were then performed to introduce the clean in-frame deletion of *panG* into *F. tularensis* Schu S4 as previously described (30, 33).

Mouse infection. Groups of 6-week-old C57BL/6 mice were anesthetized and inoculated intranasally with 50 CFU of Schu S4 or Schu S4 Δ*panG* (FTT1388). Mice were euthanized on days 1 and 3 postinfection, and lungs, livers, and spleens were aseptically removed and homogenized into 2 ml of sterile PBS using a Biojector (BIOSPEC Products, Inc.). Bacterial CFU for each organ were enumerated by plating serial dilutions of tissue homogenates onto chocolate agar. The infection experiments were approved and performed according to the animal care and use guidelines established by IACUC.

RESULTS

Organization of the *Francisella* pantothenate biosynthesis pathway. The advances in whole-genome sequencing have made identification of metabolic pathways through the use of sequence homology common. However, little experimental evidence is associated with these annotations, and gaps exist in many annotated metabolic pathways, including those of *F. tularensis* and *F. novicida*. Bioinformatics analysis suggests that the pantothenate biosynthesis pathway in *F. novicida* is much like that in *E. coli*, putatively using PanB, PanD, PanC, and IlvC to convert L-aspartate and 2-oxoisovalerate into pantothenate (Fig. 1A). Using *F. novicida* BLASTP (NCBI) generated E values of 7e10−69 for PanB, 6e10−58 for PanC, 3e10−20 for PanD, and 8e10−22 for IlvC relative to the characterized *E. coli* K-12 enzymes (34). Interestingly, no homolog of *E. coli* PanE was identified in any of the 36 sequenced *Francisella* genomes. In addition, the *ilvC* gene in *F. tularensis* Schu S4 contains a frameshift mutation that creates a premature stop codon after amino acid 74, suggesting a gap in the pantothenate biosynthetic pathway. LVS also has a truncation in the *panD* gene, shortening the encoded protein from 111 amino acids to 91 amino acids.

Unlike those of *E. coli*, the *panB*, *panC*, and *panD* genes of *Francisella* species are organized into a predicted operon (Fig. 1B and C), which is conserved in all the sequenced *Francisella* strains (35). In addition to *panBCD*, this putative operon contains additional predicted open reading frames (ORFs) both 5′ and 3′ of *panBCD* (Fig. 1C). There is an annotated ORF (*panG*) immediately 5′ of *panBCD* that encodes an uncharacterized conserved protein containing a domain of unknown function, DUF2520 (36). The ORF 3′ of *panBCD* encodes a putative type III pantothenate kinase (CoaX), which catalyzes the first dedicated step in CoA synthesis from pantothenate (37, 38). *Francisella tularensis* also contains another *coaX* homolog, FTT0112, which is not part of the putative operon.

The *Francisella panBCD* genes are required for growth in the absence of pantothenate. We used the *F. novicida* comprehensive two-allele transposon mutant library to assess the functionality of individual components of the pantothenate biosynthesis pathway (24). The pantothenate biosynthetic pathway in *F. novicida* was characterized by complementing auxotrophic mutants with metabolic intermediates of pantothenate biosynthesis.

FTN_1352 (PanB) is a ketopantoate hydroxymethyl transferase that catalyzes the conversion of 2-oxoisovalerate to 2-dehydropantoate (Fig. 1A). Functional inactivation of *panB* results in mutant strains deficient in 2-dehydropantoate and loss of pantothenate synthesis. The *panB*::Tn mutant exhibited limited growth in CDM lacking pantothenate, and growth of this mutant was restored by supplementation with pantolactone (an alternative substrate to pantoate) (Fig. 2A), thereby bypassing the PanB/IlvC branch (31). Supplementation with β-alanine alone was not sufficient to support growth of this mutant (Fig. 2A), demonstrating that FTN_1352 functions in the PanB/IlvC branch of the pathway and is consistent with the annotation of FTN_1352 as *panB*.

FTN_1353 (PanC) encodes pantothenate synthase, which catalyzes the formation of pantothenate from L-pantoate and β-alanine (Fig. 1A). Inactivation of *panC* results in a strain that requires the addition of pantothenate for growth, and neither supplementation with pantolactone nor β-alanine rescued growth. This indicates that the FTN_1353 transposon mutation interrupts the pantothenate biosynthesis pathway downstream of both the PanD and PanB/IlvC branches, consistent with the phenotype of a pantothenate synthase mutant (Fig. 2B).

FTN_1354 (PanD) is an L-aspartate α-decarboxylase that forms β-alanine from L-aspartate (Fig. 1A), and inactivation of *panD* results in a strain that is auxotrophic for β-alanine. Consistent with this phenotype, the *panD*::Tn mutant exhibited growth defects in CDM lacking pantothenate and was rescued by the addition of β-alanine. Supplementation with pantolactone failed to restore growth of the *panD*::Tn mutant (Fig. 2C). These results demonstrate that inactivation of FTN_1354 results in a loss of

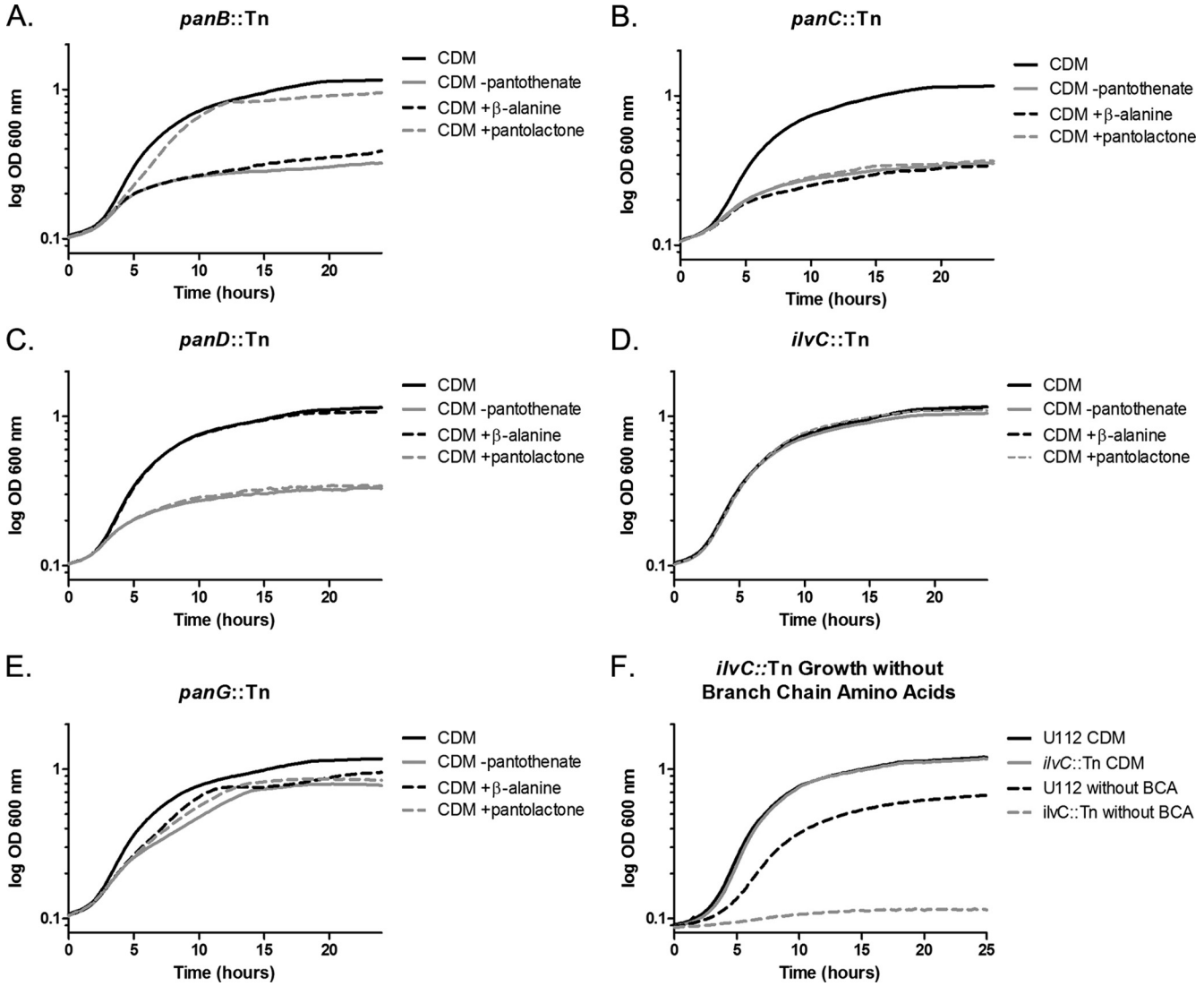

FIG 2 *F. novicida* functional complementation of *panB*::Tn, *panC*::Tn, *panD*::Tn, *ilvC*::Tn, and *panG*::Tn. Functional complementation of the pantothenate biosynthetic genes in *F. novicida* transposon mutant strains grown in 96-well microtiter plates with absorbance (OD_{600}) monitored every 15 min in CDM lacking pantothenate (gray) either supplemented with β-alanine (black dotted line), pantolactone (gray dotted line), or calcium pantothenate (black). Shown are growth curves for *panB*::Tn (A), *panC*::Tn (B), *panD*::Tn (C), *ilvC*::Tn (D), and *panG*::Tn (E) mutants. (F) Growth of *F. novicida* U112 and *ilvC*::Tn in CDM with and without branch chain amino acids: *F. novicida* U112 in CDM (black), *ilvC*::Tn in CDM (gray), U112 in CDM without branch chain amino acids (black dotted line), and *ilvC*::Tn in CDM without branch chain amino acids (gray dotted line). Each growth curve was repeated a minimum of three times, and the graph represents the means of three replicate experiments.

β-alanine synthesis and are consistent with the prediction that FTN_1354 is a functional PanD (4).

FTN_1040 (IlvC) is known to function as an acetohydroxy acid isomerase essential for branch chain amino acid synthesis and can also function as a KPR in *E. coli* and *S.* Typhimurium (39). As the only recognized enzyme with KPR activity within the *F. novicida* genome, we were surprised to find that the *ilvC*::Tn transposon mutant in *F. novicida* did not require pantothenate for growth (Fig. 2D). This result suggested that there was another unrecognized enzyme with sufficient KPR activity to support pantothenate biosynthesis within *F. novicida ilvC*::Tn.

FTN_1351 (*panG*) is annotated as an uncharacterized conserved gene and is part of the putative pantothenate operon, so we tested the *panG*::Tn mutant's pantothenate growth requirement. Under each condition, the *panG*::Tn mutant grew, suggesting that either PanG may not be involved in pantothenate synthesis or it is functionally redundant with IlvC in KPR activity (Fig. 2E).

To determine if IlvC in *F. novicida* is a functional acetohydroxy acid isomerase, we grew wild-type *F. novicida* U112 and *ilvC*::Tn in CDM with and without branch chain amino acids (Fig. 2F). Only wild-type U112 could grow in CDM without branch chain amino acids, supporting our hypothesis that IlvC functions as an acetohydroxy acid isomerase in *F. novicida*.

New class of ketopantoate reductase found in a number or pathogenic organisms. Ketopantoate reductases (KPR) have a dinucleotide-binding domain called a Rossmann-fold that consists of a βαβ pocket to accommodate the coenzyme NADPH (40). Within the Rossmann-fold is a consensus sequence, GXGXXG,

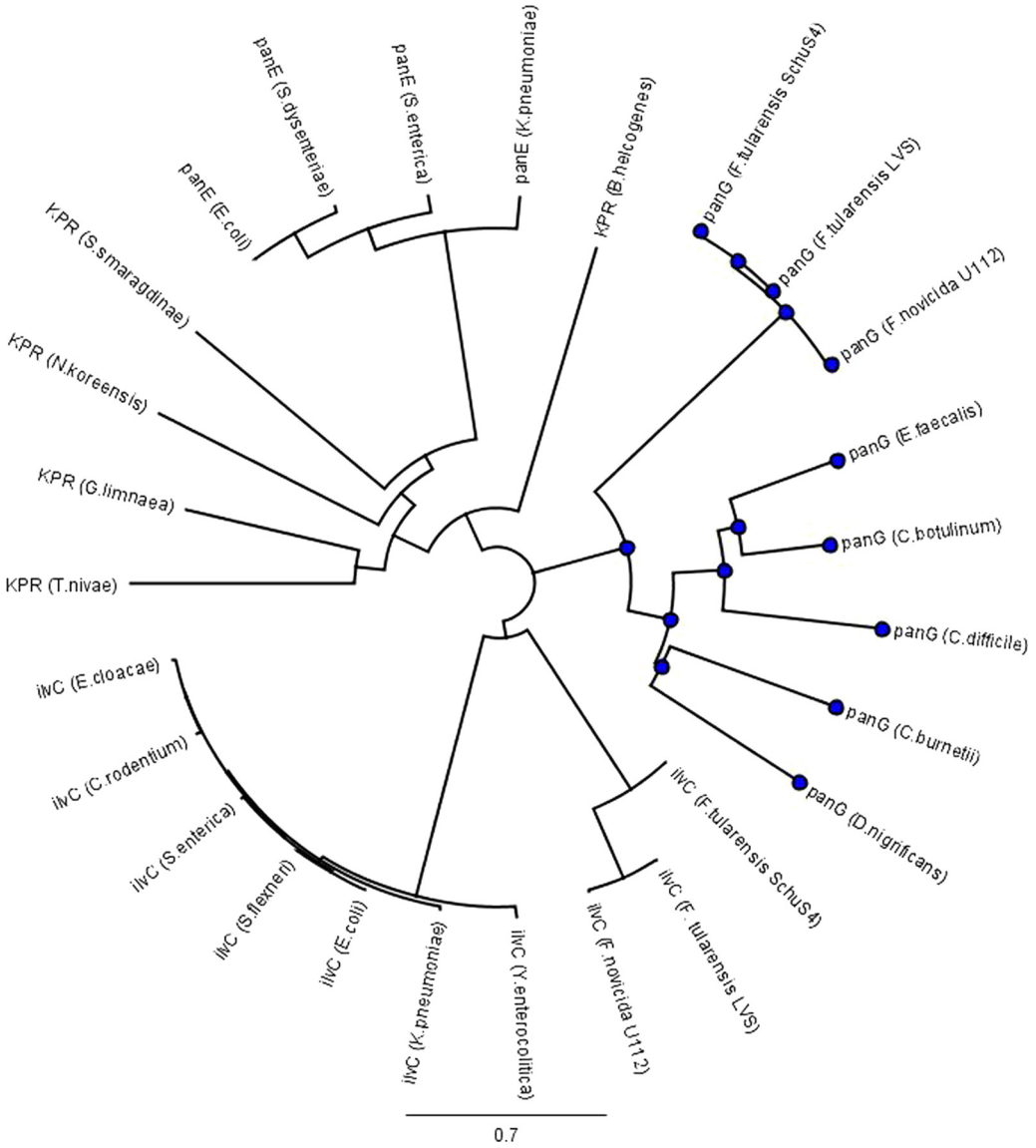

FIG 3 Phylogenetic tree of known ketopantoate reductase proteins and PanG. The phylogenetic tree of known KPR proteins and PanG was generated using Geneious Pro 5.5.6 Tree Builder with the cost matrix set to identity and Jukes-Cantor as the genetic distance model with no outgroups. All IlvC and PanE proteins are annotated on PubMed to be involved in pantothenate synthesis, while all PanG proteins are annotated as hypothetical proteins. *Enterococcus faecalis* V583 PanG is annotated as hypothetical protein EF1861, *Clostridium difficile* 630 PanG is annotated as hypothetical protein CD630-15140, *Coxiella burnetii* RSA 493 PanG is annotated as hypothetical protein CBU_1660, *Clostridium botulinum* BKT015925 PanG is annotated as hypothetical protein CbC4_0183, and *Desulfotomaculum nigrificans* DSM 574 PanG is annotated as hypothetical protein DUF2520.

which appears at the border between the first beta sheet and the alpha helix (41). In *E. coli* the PanE N-terminal Rossmann-fold domain and the C-terminal alpha-helical domain form a cleft, and at that cleft is a conserved essential residue, Lys176, that forms the hydrogen bond with C_2 hydroxyl of pantoate (8). Pantoate also forms additional interactions with conserved residues Ser244, Asn98, and Asn180 (8). PanE and IlvC each have an N-terminal NADB_Rossmann superfamily domain (36). IlvC is an acetohydroxy acid isomerase, which can also function as a KPR and requires two cofactors, NADPH and Mg^{2+}, for the reduction of 2-dehydropantoate (42).

Bioinformatic analysis of PanG (FTN_1351) gave little insight into its function. The comparison of the sequence to PanE and other known KPR revealed no significant matches (5, 43). Phylogenetic analysis indicated that PanG is in a distinct group separate from annotated KPRs using the Jukes-Cantor genetic distance model (Fig. 3). There is no Rossmann domain identified in the C-terminal region of FTN_1351, and the GXGXXG motif is not present (41). The C-terminal region of FTN_1351 has a domain of unknown function, DUF2520 (E value of 1.5e10−6), that often accompanies an N-terminal Rossmann domain, suggesting that the protein could function as an uncharacterized KPR (36). We made a double mutation, *ilvC*::Tnflp *panG*::Tn, in *F. novicida* to determine if FTN_1351 protein could function as a KPR. The double mutant did not grow in medium lacking pantothenate (Fig. 4A). Growth of the double mutant was restored when com-

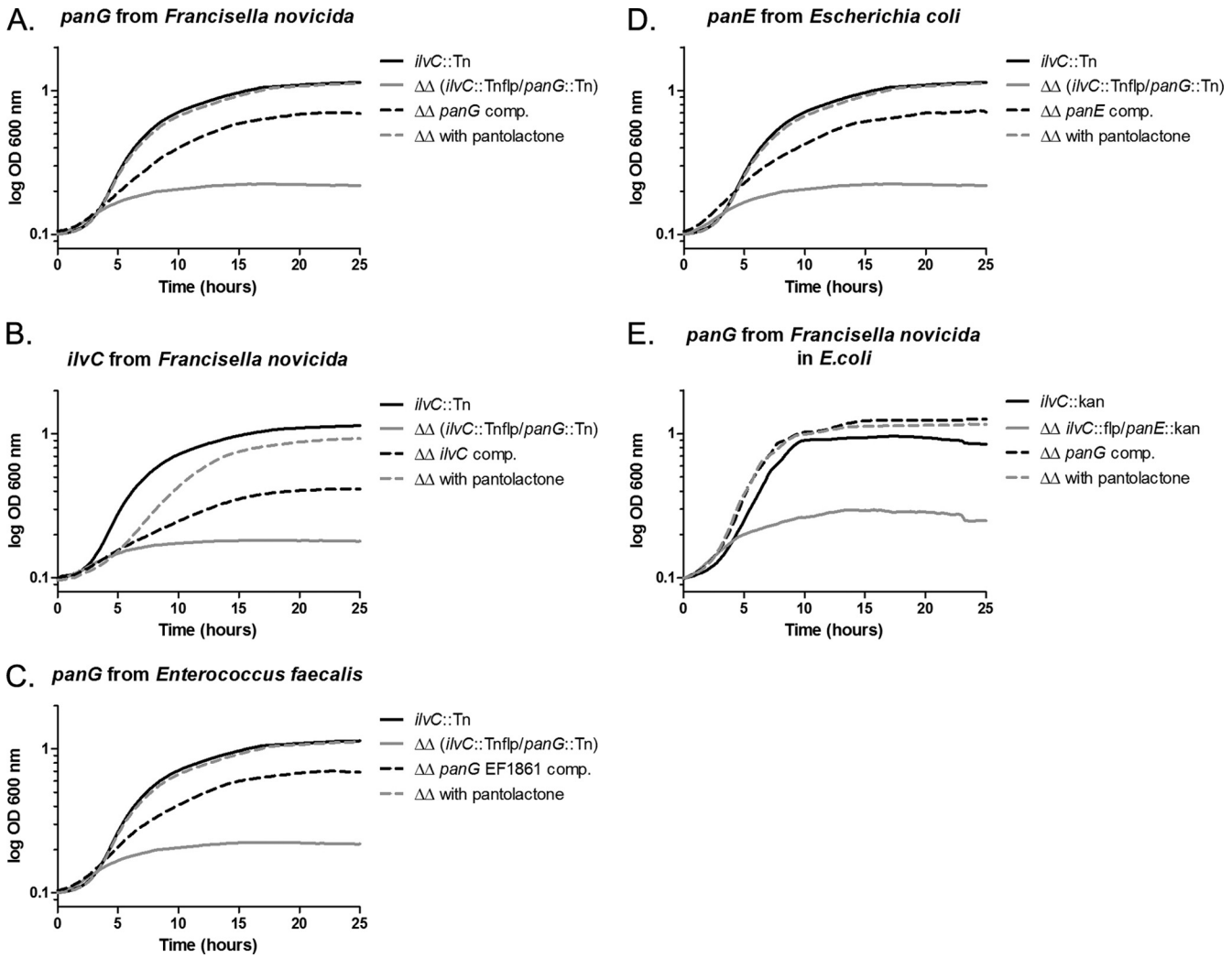

FIG 4 Genetic complementation of the *F. novicida ilvC*::Tnflp *panG*::Tn double mutant with *F. novicida* FTN_1351 *panG*, *F. novicida ilvC*, *E. faecalis* V583 (EF1861) *panG*, and *E. coli panE* and genetic complementation of the *E. coli ilvC*::Flp *panE*::Kan double mutant with *F. novicida panG*. Functional complementation experiments were carried out by growing *Francisella* in CDM lacking pantothenate in 96-well microtiter plates and measuring the absorbance (OD_{600}) every 15 min for 30 h. Competent *F. novicida* isolates were transformed with DNA. (A) pSKI01 carrying *F. novicida panG* (FTN_1351) driven by its native promoter; (B) pSKI04 carrying *ilvC* from *F. novicida* driven by *F. tularensis blaB* promoter; (C) pEDL71 carrying *panG* from *E. faecalis* driven by *F. tularensis blaB* promoter; (D) pEDL70 carrying *panE* from *E. coli* driven by *F. tularensis blaB* promoter. *ilvC*::Tn mutant grown in CDM lacking pantothenate containing empty control vector pMP822/pMP831 (black), *ilvC*::Tnflp *panG*::Tn double mutant containing an empty control vector (gray), *ilvC*::Tnflp *panG*::Tn double mutant containing the respective complementing plasmid (black dotted line), and *ilvC*::Tnflp *panG*::Tn double mutant grown in CDM supplemented with pantolactone (gray dotted line). (E) *E. coli ilvC*::Flp *panE*::Kan double mutant complemented with pSKI01 carrying *F. novicida panG* driven by its native promoter. *ilvC*::Kan mutant grown in M9 medium lacking pantothenate containing an empty control vector (black), *ilvC*::Flp *panE*::Kan double mutant containing an empty control vector (gray), *ilvC*::Flp *panE*::Kan double mutant containing the respective complementing plasmid (black dotted line), and *ilvC*::Flp *panE*::Kan double mutant grown in M9 supplemented with pantolactone (gray dotted line). Each growth curve experiment was repeated three times, and the graph represents the mean of three replicate experiments.

plemented in *trans* with *panG* expressed under its native promoter, with *ilvC* driven by the *Francisella blaB* promoter or when the media was supplemented with pantolactone (Fig. 4A and B). This result confirms that IlvC is active in the *F. novicida* pantothenate metabolic pathway and that FTN_1351 is a novel KPR gene, which we named *panG*. Homologs of PanG are also found in other pathogenic bacteria, including *Enterococcus faecalis*, *Coxiella burnetii*, and *Clostridium difficile*. BLASTP analysis of *F. tularensis* Schu S4 PanG (FTT1388) revealed that this protein has significant homology with *Enterococcus faecalis* V583 (EF1861) (E value = 2e10−11), *Coxiella burnetii* (CBU1660) (E value = 2e10−7), and *Clostridium difficile* (CD630-15140) (E value = 8e10−6) (34). In *E. faecalis* and *C. difficile*, the *panG* genes are organized similarly in putative pantothenate operons (Fig. 1B). Expression of the *E. faecalis* V583 gene in *F. novicida ilvC*::Tnflp *panG*::Tn restored growth of this mutant strain to near-wild-type levels in CDM lacking pantothenate, confirming genetically that EF1861 is a functional KPR (Fig. 4C). PanE, an analog KPR from *E. coli* (EC 1.1.1.169), is not present in any sequenced genome of *Francisella*, and expression of this gene restored growth of *F. novicida ilvC*::Tnflp *panG*::Tn in medium lacking pantothenate (Fig. 4D). Expression of the *F. novicida panG* gene in the *E. coli* KPR *ilvC*::flp

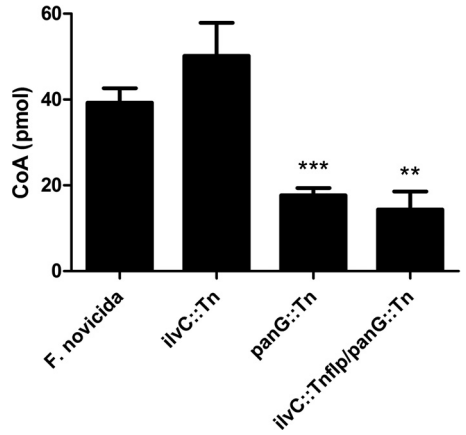

FIG 5 *F. novicida* CoA levels. CoA concentrations were measured from 50-ml cultures of *F. novicida* wild-type, *ilvC*::Tn, *panG*::Tn, and *ilvC*::Tnflp *panG*::Tn strains after 5 h of pantothenate depletion. All strains were grown to the same OD_{600} and were normalized to total protein. The CoA levels represented are the means ± standard deviations (SD) for three independent experiments. Statistical significance was determined by comparing the mutant values to those of wild-type *F. novicida*. ***, $P < 0.0001$; **, $P < 0.001$.

panE::kan double mutant restored growth in M9 minimal medium lacking pantothenate, confirming genetically that PanG is a functional KPR in *E. coli* (Fig. 4E).

***Francisella novicida* CoA levels.** Coenzyme A is made from pantothenate, cysteine, and adenosine and is an essential cofactor in the first step of the tricarboxylic acid (TCA) cycle. To determine the contribution of PanG and IlvC in the production of pantothenate and subsequent CoA synthesis, we measured the concentration of CoA after 5 h of pantothenate depletion in wild-type *F. novicida* and in the *ilvC*::Tn, *panG*::Tn, and *ilvC*::Tnflp *panG*::Tn mutants. CoA levels were not significantly different between the wild type and the *ilvC*::Tn mutant, suggesting that PanG can fulfill the requirement for KPR activity in *F. novicida* (Fig. 5). The CoA levels of the *panG*::Tn mutant strain were less than one-half of wild-type levels and similar to levels of the *ilvC*::Tnflp *panG*::Tn double mutant, suggesting that PanG is responsible for the majority of KPR activity in *F. novicida* (Fig. 5).

***Francisella tularensis* LVS is a β-alanine auxotroph.** Early studies revealed various nutritional requirements of *Francisella* strains for pantothenate that appeared to correlate with virulence in mice (9, 10). We assessed the requirement for pantothenate with three different strains of *Francisella*: *F. tularensis* Schu S4, *F. tularensis* LVS, and *F. novicida* U112. Each strain grew logarithmically in complete CDM (Fig. 6A to D). *F. tularensis* Schu S4 and *F. novicida* U112 grew logarithmically under each dropout condition, suggesting that both strains have complete pantothenate biosynthesis pathways (Fig. 6). However, *F. tularensis* LVS failed to grow in medium without pantothenate, indicating that LVS is a pantothenate auxotroph. This result is in agreement with the observations of Chamberlain (10). Additionally, we observed that growth of LVS could be restored by the addition of β-alanine, indicating that LVS lacks PanD activity (Fig. 6D). Comparison of the nucleotide sequences of the putative *panGBCD* genes among *Francisella* species revealed that *F. tularensis* LVS contains a base substitution in the annotated *panD* gene. This substitution creates a Q92 Ochre stop, resulting in a truncation of PanD by 20 amino acids. To determine if LVS β-alanine auxotrophy is due to a nonfunctional *panD*, we replaced $panD_{LVS}$ with the functional

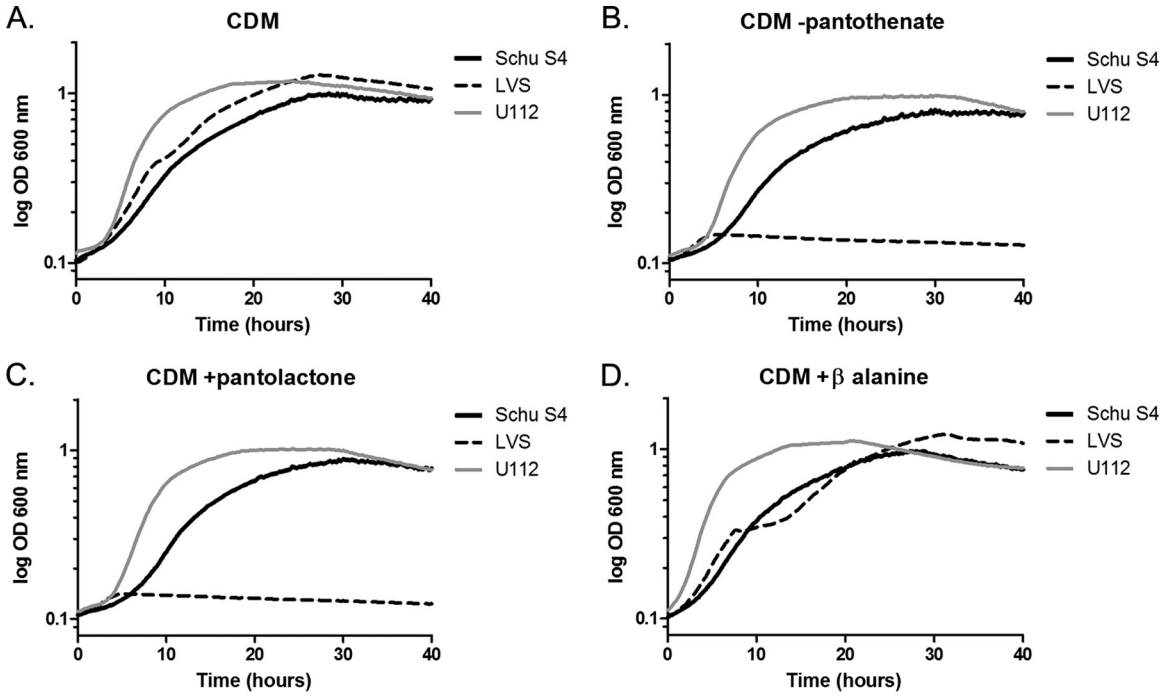

FIG 6 *F. tularensis* Schu S4, *F. tularensis* LVS, and *F. novicida* growth in pantothenate dropout media. Growth curves of *F. tularensis* subsp. *tularensis* Schu S4 (black), *F. tularensis* subsp. *holarctica* LVS (black dotted line), and *F. novicida* U112 (gray) were monitored in CDM (A), CDM lacking pantothenate (B), CDM lacking pantothenate supplemented with pantolactone (C), or CDM lacking pantothenate supplemented with β-alanine (D). Each strain was grown in triplicate in 96-well microtiter plates with absorbance (OD_{600}) monitored every 15 min over 40 h. Each graph represents the mean of three replicate experiments.

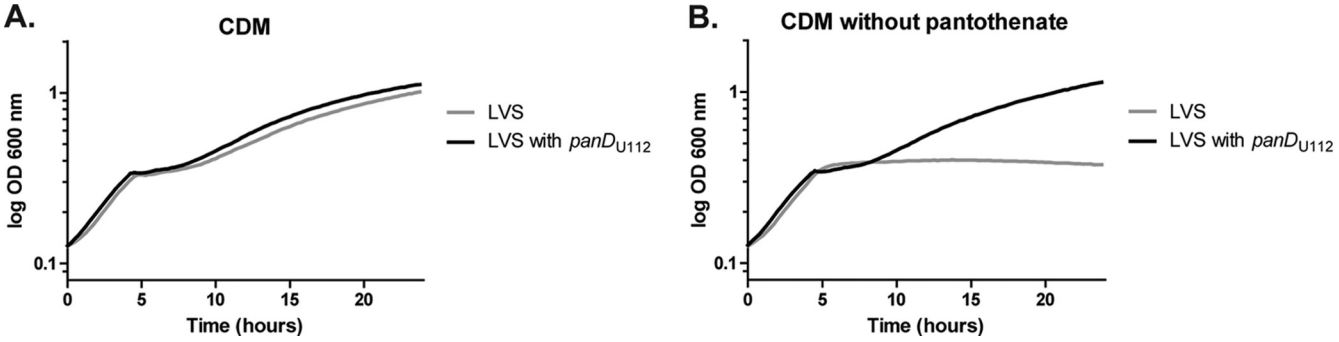

FIG 7 Repair of *F. tularensis* LVS β-alanine auxotrophy. Growth curves of LVS (gray) and LVS with the native $panD_{LVS}$ replaced with $panD_{U112}$ from *F. novicida* U112 (black). Each strain was monitored in CDM (A) or CDM lacking pantothenate (B). Both strains were grown in 96-well microtiter plates, and the OD_{600} was determined every 15 min for 24 h. Each graph represents the mean OD_{600} of three replicate experiments.

$panD_{U112}$ via allelic exchange. As predicted, *F. tularensis* LVS harboring the $panD_{U112}$ allele grew similarly in CDM with or without added pantothenate (Fig. 7). This demonstrates that the observed pantothenate auxotrophy of LVS results from a lack of PanD activity. This truncation in PanD is unique to LVS, so we questioned whether the lack of PanD activity contributed to the attenuated phenotype of this strain. However, repair of *panD* activity in *F. tularensis* LVS $panD_{U112}$ did not confer a competitive advantage relative to *F. tularensis* LVS $panD_{LVS}$ in a mouse model of pneumonic tularemia (data not shown). Thus, the LVS *panD* mutant allele does not contribute to the attenuated phenotype of this strain.

***Francisella tularensis* Schu S4 Δ*panG* is a pantothenate auxotroph.** The Δ*panG* (FTT1388) strain in Schu S4 requires pantothenate for growth and can be rescued with the addition of pantolactone in CDM lacking pantothenate or with *panG* expressed in *trans*. Schu S4 does not have a functional IlvC protein, at least in regard to its KPR activity, due to a frameshift mutation, making PanG the sole KPR. The Δ*panG* strain can be complemented in *trans* with the *panG* gene from *F. novicida* driven by the *F. novicida* native promoter (Fig. 8B). Given that *F. novicida panG* (FTN_1351) was identified in a screen to be required for virulence in a mouse model of infection (21) and *F. tularensis panG*, *panB*, *panC*, *panD*, and *coaX* genes were highly upregulated within bone marrow-derived macrophages, pantothenate production and ultimately CoA synthesis may play a role in adaptation of *Francisella* to its intracellular niche (22). We wanted to determine if the Δ*panG* mutation was important for infection in mice. With an intranasal inoculum of 50 CFU, we saw no difference in lung, liver, or spleen burdens when comparing Δ*panG* to wild-type Schu S4 at 1 or 3 days postinoculation (Fig. 8C to E).

DISCUSSION

Pantothenate forms the core of CoA and is a precursor to acyl carrier protein (ACP), making it essential in both energy and lipid metabolism. We characterized the genes that function in pantothenate biosynthesis in *Francisella* using genetic and chemical complementation approaches. These genes are organized into a putative operon, which also included a gene coding for a hypothetical protein, PanG (FTN_1351), which we discovered is a novel KPR responsible for converting 2-dehydropantoate to pantoate. PanG (FTN_1351) does not have the known conserved Rossmann-domain that is typically associated with KPRs. However, PanG does contain a C-terminal DUF2520, which usually accompanies an N-terminal Rossmann-like domain. PanG rescued growth of an *E. coli* KPR double mutant in the absence of pantothenate, and the *panG* homolog from *E. faecalis* V583 (EF1861) complemented KPR function in the *F. novicida* double mutant. EF1861 also contains a predicted C-terminal DUF2520 domain in addition to a predicted N-terminal Rossmann-like domain. The *panG* genetic data (FTN_1351 and EF1861) suggest that the DUF2520 domain is involved in KPR activity. Further biochemical characterization of PanG needs to be done to determine structural binding motifs and enzymatic activity. Normally, KPR proteins bind cofactors such as NAD or NADP; more work needs to be done to characterize what cofactor is required for PanG KPR activity. There is no GXGXXG motif in PanG, which is responsible for NADP binding in *E. coli*; however, amino acids 11 to 16 are GXGXXA, which is a sequence found in mitochondrial flavoenzymes that bind NADP (41). Using secondary-structure predictions of PanG, the GXGXXA consensus sequence starts right at the junction of a β-sheet and an α-helix that would be compatible with a KPR βαβ fold-forming sequence (44).

Functional complementation assays demonstrated that expression of *panB*, *panC*, and *panD* were each required for growth of *F. novicida* in medium devoid of pantothenate. In regard to KPR activity, only an *ilvC*::Tnflp *panG*::Tn double mutant of *F. novicida* created a pantothenate auxotroph, demonstrating that PanG is functionally redundant with the acetohydroxy acid isomerase, IlvC. Both the *panG* homolog from *E. faecalis* V583 and the known *panE* gene encoding a KPR from *E. coli* DH10B could functionally complement the *F. novicida ilvC*::Tnflp *panG*::Tn KPR double mutant. CoA levels were not significantly different between the wild type and the *ilvC*::Tn mutant after 5 h of pantothenate depletion, demonstrating that PanG can fulfill the requirement for KPR activity in *F. novicida*. CoA levels in both the *panG*::Tn mutant strain and the *ilvC*::Tnflp *panG*::Tn double mutant were less than one-half the concentration of the wild type, suggesting that PanG is responsible for the majority of KPR activity in *F. novicida*. We also found that the LVS strain is a β-alanine auxotroph resulting from a base substitution in *panD* causing an amino acid substitution that shortens the aspartate-1-decarboxylase. While the lack of PanD activity appears to be unique to the LVS strain, it does not contribute to the attenuation of this strain in mice. In addition, the Schu S4 frameshift in *ilvC* produced a nonfunctional protein in regard to KPR activity. We demon-

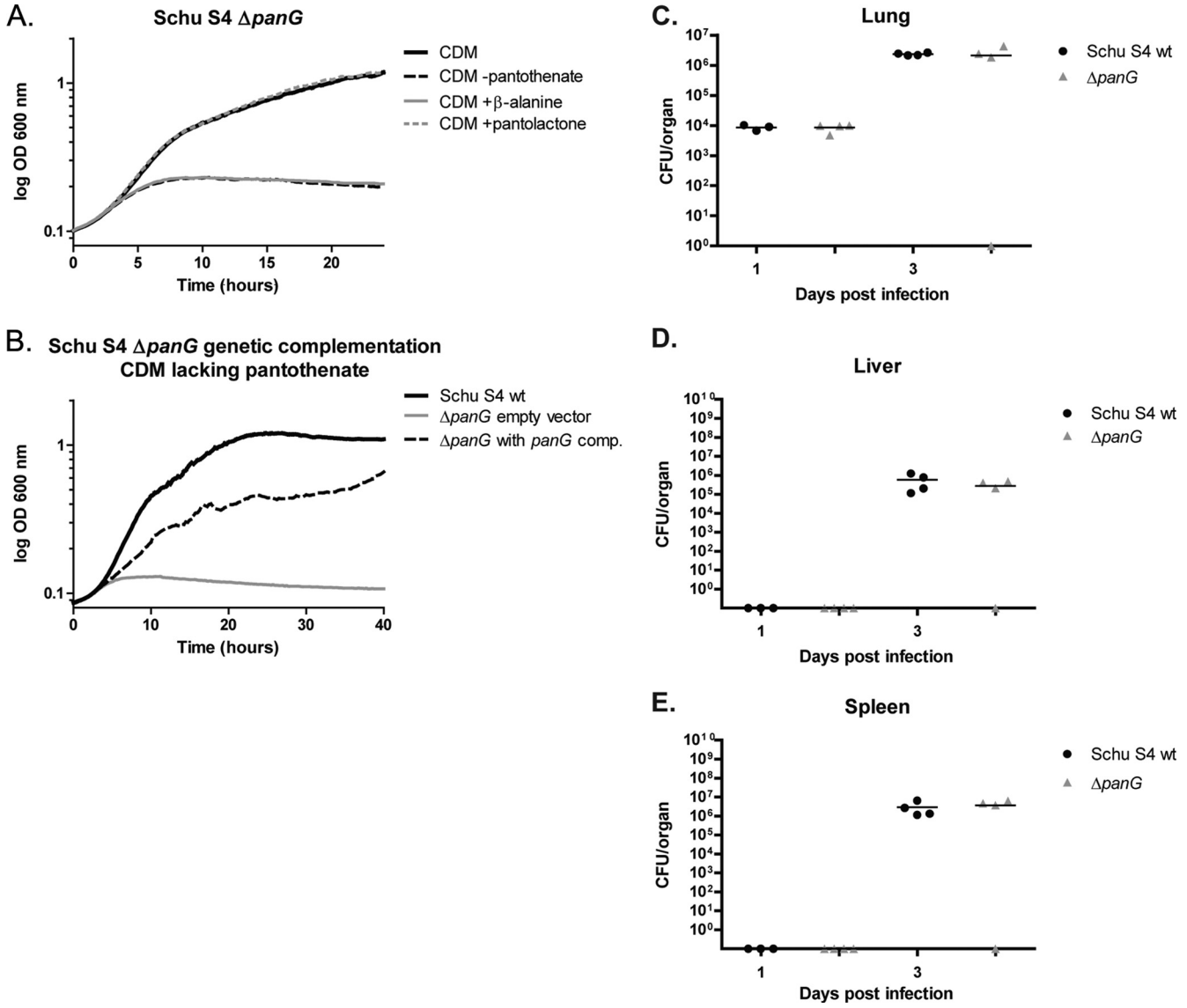

FIG 8 *F. tularensis* subsp. *tularensis* Schu S4 Δ*panG* growth and virulence phenotype. (A) Chemical complementation of Schu S4 Δ*panG* grown in CDM (black), CDM lacking pantothenate (black dotted line), CDM lacking pantothenate supplemented with β-alanine (gray), and CDM lacking pantothenate supplemented with pantolactone (gray dotted line). (B) Genetic complementation of *F. tularensis* subsp. *tularensis* Schu S4 Δ*panG* with *F. novicida panG* expressed by the native promoter in the shuttle vector pMP831. Growth curve of *F. tularensis* subsp. *tularensis* Schu S4 (black), *F. tularensis* subsp. *tularensis* Schu S4 Δ*panG* with pMP831 (empty control vector) (gray), and *F. tularensis* subsp. *tularensis* Schu S4 Δ*panG* complemented with pSKI01 (black dotted line). All growth curves were done in triplicate, monitoring absorbance (OD_{600}) in a 96-well microtiter dish. Graphs represent the mean absorbance at OD_{600}. (C to E) Recovery of Schu S4 or Schu S4 Δ*panG* mutant in mice following intranasal inoculation. C57BL/6 mice were infected intranasally with either wild-type Schu S4 (black circles) or Schu S4 Δ*panG* (gray triangles) at a lethal dose of 50 CFU. Mice were euthanized on days 1 and 3 postinfection, and lung burdens (C), liver burdens (D), and spleen burdens (E) were determined and graphed. Each symbol represents data from a single mouse. There were no significant differences in recovery of mutant versus wild-type organisms from any organ at any time point as determined by the nonparametric Mann-Whitney test.

strated that PanG is the sole KPR in Schu S4, since creating an in-frame *panG* deletion resulted in a pantothenate auxotroph. However, the Schu S4 Δ*panG* strain did not have a demonstrable virulence defect in a mouse model of pneumonic tularemia. It is not clear what molecule this mutant acquired from the host or where it acquired it to satisfy its pantothenate requirement. Given that mice have a blood plasma pantothenate level of 20 μM while humans have only 2 to 4 μM, it is possible that an *F. tularensis* pantothenate synthesis mutant could be attenuated in humans but not mice (45). It is possible that mice have excess pantothenate in their bloodstream, considering their diet is supplemented with pantothenate in addition to that naturally acquired from their gut flora. *E. coli* can produce and secrete 15 times more pantothenate than required for CoA biosynthesis, and ruminants obtain sufficient quantities of pantothenate from their gut microorganisms (46, 47). Virtually all bacteria, including *F. tularensis*, can take up pantothenate, but *F. tularensis* does not have an annotated *panF* homolog responsible for sodium-cotransport of pantothenate (1). It appears that *F. tularensis* is able to obtain pantothenate, a CoA precursor (such as phosphopantetheine), or CoA from the host

and is likely storing sufficient quantities of pantothenate to support logarithmic growth for several rounds of replication while inside cells, where pantothenate may be limited. Pantothenate is immediately phosphorylated once taken up by host cells and could affect the ability of *F. tularensis* to acquire pantothenate. Further work will be needed to determine how *F. tularensis* acquires pantothenate or other substituents of CoA from the host. It may be a valuable endeavor to look further down in the CoA pathway for potential drug targets for *Francisella tularensis*, starting with the putative type III pantothenate kinases (FTT1392 and FTT0112), which is the first committed step in CoA biosynthesis.

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